

**AN AGED RODENT MODEL OF STROKE RECOVERY:  
EXAMINING THE ROLE OF THE MEDIAL PREFRONTAL CORTEX**

by

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## **Abstract**

Covert and recurrent strokes increase during aging, emphasizing the importance of using aged rodent models of recurrent stroke.

In experiment 1, 25 young and 16 middle-aged Sprague Dawley rats underwent medial prefrontal cortex (mPFC) or sham ischemia. There was no overt motor effect of mPFC ischemia, making it a valid model of covert stroke. Notably, middle-aged rats showed decreased overall performance in the staircase reaching test, relative to younger rats.

In experiment 2, 34 young and 33 middle-aged rats underwent middle cerebral artery occlusion (MCAo) +/- mPFC ischemia. Unlike experiment 1, there was no effect of age on baseline performance. The middle-aged group, however, had less motor impairments and significantly smaller striatal infarcts, suggesting the MCAo strokes were suboptimal.

These results highlight important age-related differences in post-stroke performance. Behavioural outcome measures, motivation level and the ischemic model must be carefully considered in developing older animal models of stroke.

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**List of Abbreviations**

ANOVA.....analysis of variance

AP.....anteroposterior

Bilat.....bilateral

DV.....dorsoventral

ET-1.....Endothelin-1

H&E.....hematoxylin and eosin

Hem.....hemisphere

Isch.....ischemia

MCA.....middle cerebral artery

MCAo.....middle cerebral artery occlusion

mg.....milligram

Mid-age.....middle-aged

ML.....mediolateral

mm.....millimeter

mPFC.....medial prefrontal cortex

MRI.....magnetic resonance imaging

PBS.....phosphate buffered saline

PFC.....prefrontal cortex

pmol.....picamol

PS.....post-stroke

RAM.....radial arm maze

tPA.....tissue-type plasminogen activator

Unilat.....unilateral

$\mu$ l.....microliter

$\mu$ m.....micrometer

## **1. Introduction**

Stroke is a leading cause of death and disability worldwide, affecting an estimated 405,000 Canadians who are currently living with the lasting effects of stroke (Krueger et al., 2015). Of these individuals, at least 40% experience moderate to severe impairments that negatively impact their daily lives, and 80% have restrictions to their daily activities secondary to stroke (Canadian Stroke Network, 2011; Public Health Agency of Canada, 2011). In addition to the obvious personal implications of stroke and the psychological and social burden on survivors and their families, the financial cost for care of these survivors is well over \$3 billion per year (Public Health Agency of Canada, 2011). This cost accounts for health care as well as lost productivity due to premature death and disability. Additionally, the latest projections estimate that the incidence of stroke in Canada will increase even further, by up to 80% by 2038 (Krueger et al., 2015). This is due to a number of factors including aging, an increase in the Canadian population, and the detrimental effects of a more sedentary lifestyle and unhealthy diet. This of course will place enormous demands on our health care system.

Stroke is caused by an interruption of circulation to a localized part of the brain, following an obstructive (thrombotic or embolic) or hemorrhagic event. When this interruption in brain perfusion (termed ischemia) endures for a sufficient period of time it causes irreparable tissue damage and cell death. Ischemic injury may then produce a variety of functional impairments that include impaired motor, sensory and cognitive performance, depending upon the affected area. Ischemic stroke represents 80-85% of stroke cases in Canada, while a rupture of a major brain artery (hemorrhage) accounts for the remaining 15-20% of stroke cases (Mozzafarian et al., 2016).

In recent years, there has been growing awareness of another form of stroke called “covert” stroke, appropriately termed because unlike obvious “overt” impairments such as motor, sensory, language and speech deficits, the impairments arising from covert stroke are not obvious, often affecting cognitive function and progressing over time to vascular cognitive impairment (Jellinger 2013; Iadecola 2013). Overt strokes primarily affect the large arteries of the brain (ie. middle cerebral artery, MCA) and result in large areas of damage or infarcts, easily appreciated on brain imaging. In contrast, covert strokes are due to small vessel disease, and the damage to white matter tracts appear as hyperintensities on magnetic resonance imaging (MRI), and are often difficult to appreciate depending on the imaging modality used. Small areas of subcortical tissue damage, termed lacunes, are associated with covert strokes, and are now known to have a much higher prevalence than what was previously appreciated (Vermeer et al., 2007). The identification of these lacunes are not only an indicator of underlying vascular disease and a marker for progressive cognitive impairment, but their presence also more than doubles an individual’s risk of subsequent stroke (Vermeer et al., 2007).

There are limited treatment options available for those who present during the acute phase of a stroke. This includes pharmacological management in the setting of an ischemic stroke due to thrombus or emboli, and neurosurgical and endovascular interventions in a very small and select group of patients. No matter the mechanism of stroke, it is crucial that medical attention is sought soon after the onset of symptoms, as the treatment options, if any, are effective only during a limited period of time. This requires early recognition of symptoms by the individual and/or those in their company, prompt access to a healthcare facility that is able to conduct the appropriate investigations and provide the necessary interventions in a timely manner, and good baseline health status with no preexisting medical conditions or

contraindications to using the available interventions. In the United States, this has meant that over 90% of stroke patients do not meet criteria, and are therefore unable to receive acute intervention (Nasr et al., 2013).

The sole medication currently proven to favourably affect outcomes when given during the acute phase of ischemic stroke is recombinant tissue-type plasminogen activator (tPA), and the best surgical option at present is endovascular clot retrieval. Both interventions are limited in their clinical application and availability for a number of reasons. Tissue-type plasminogen activator is most effective when used within 3 hours of symptom onset, but can be used up to 4.5 hours after symptoms begin in a slightly more select population (Demaerschalk et al., 2016). Even in those with major stroke symptoms, it is estimated that less than 10% of ischemic stroke patients have no contraindications and are eligible to receive t-PA (Kleindorfer et al., 2004; de los Rios la Rosa et al., 2012; Demaerschalk et al., 2016; Canadian Stroke Network Statistics, 2011).

The use of stent retriever mechanical thrombectomy is the most novel therapy currently available, yet its use is also limited, again, as many patients do not meet the criteria for intervention. In order to undergo thrombectomy, there must be evidence of proximal occlusion of the M1 (main trunk) segment of the MCA, with or without concomitant occlusion of the internal carotid artery, and the exclusion of a large core on cerebral imaging (ruling out a large cerebral infarct) (Jovin et al., 2015; Sivan-Hoffman et al., 2016; Campbell et al., 2015). This alone limits many stroke patients from receiving this potential therapy, though it has proven to be quite effective, particularly when used in combination with tPA, and can also be used at up to 8 hours after the onset of symptoms following an ischemic stroke (Jovin et al., 2015; Saver et al., 2015; Goyal et al., 2015).

Due to the limits of available interventions for the treatment of acute stroke, the vast majority of stroke patients are therefore left without an option for immediate therapy. They must instead rely on rehabilitation as the best option to improve their functional recovery. When patients experience motor and language deficits, physical therapy is started in the in-patient setting and is continued for weeks to months in an attempt to maximize recovery of function. A large number of patients experience not only lasting motor impairments, but also cognitive and emotional alterations including depression and an overall decrease in self-reported quality of life (Pendlebury et al., 2011; Fens et al. 2013; Moran et al, 2014), which is even more difficult to treat (Teasell, 2014). While beneficial, rehabilitation provides only partial motor recovery, and in most instances nearly 50% of patents have residual impairments that interfere with activities of daily living 5 years after their strokes (Teasell, 2014).

Consequently, considerable preclinical research has investigated different types, intensities, and timing of rehabilitation in attempts to optimize post-stroke recovery (Kleim & Jones, 2008; Murphy & Corbett, 2009; Krakauer et al., 2012; Wahl & Schwab, 2014). However, there is growing consensus that in order to optimize rehabilitation it is necessary to understand the biological processes contributing to recovery so that rehabilitation strategies can be directed towards biological targets and truly optimized (Corbett et al 2015; Bernhardt et al 2016; Carmichael 2016).

## **1.1 Animal Models of Stroke**

There are many models of rodent stroke, each with their own associated limitations and strengths based on the mechanism and its relevance to clinical human stroke. This includes vessel occlusion by chemically induced thromboembolism (by chemical photothrombosis or

induced emboli), by mechanical obstruction using a clip or a ligature (to a single vessel, as in the MCA occlusion (MCAo) monofilament, or to multiple vessels as is used in the three vessel occlusion model which includes occlusion of the MCA and both common carotid arteries), or using a potent vasoconstrictor (such as ET-1) injected in a corresponding brain region to induce subsequent ischemia. Each of these stroke models have been developed and refined as varying methods to occlude the MCA and more closely replicate human clinical stroke, as the MCA is that which is most affected in human ischemic stroke and is most commonly targeted in rodent stroke models (Mohr et al., 1986; Macrae, 2011). For the purposes of this particular research question, I chose to proceed with the ET-1 model for both mPFC ischemia and MCA occlusion, for reasons as described below.

First introduced by Koizumi et al. in 1986, the MCA occlusion by intraluminal filament is the most widely used model of focal ischemia in rodents, and can be used to induce either permanent or transient ischemia. The main advantage is the ability to control the duration of ischemia (most commonly 60, 90, or 120 minutes), as well as the fact that it is technically easier to master and less invasive than those models that require a craniotomy. An incision is made to locate the common carotid artery, and a flexible monofilament is passed through the internal carotid artery and advanced until it blocks the origin of the MCA and subsequently prevents perfusion (Macrae 2011). Many modifications have been made to this model, including the type of monofilament and its coating, but this model continues to have significant limitations in reproducibility as well as morbidity and mortality. Even with improvements and the use of a coated suture, this model is associated with a 12% rate of subarachnoid hemorrhage (which reduces cerebral perfusion bilaterally), and can be associated with additional areas of ischemia, including those supplied by the external carotid artery as well as the anterior choroidal and

hypothalamic arteries (Carmichael 2005; Macrae 2011). This causes unwanted ischemia to the muscles of mastication and swallowing, for example, and results in weight loss and poorer subsequent performance on behavioural outcome measures (Dittmar et al., 2003), or even hyperthermia secondary to damage to the hypothalamus (Li et al., 1999). This method is also associated with a decrease in the severity of ischemia in certain rodent strains if they have good collateral perfusion to the cortex which can compensate for the decreased perfusion by the MCA (Coyle & Jokelainen, 1983; Duverger & Mackenzie, 1988). In order to circumvent this issue, some studies use the three-vessel occlusion technique to occlude the MCA as well as both common carotid arteries, however this model produces a smaller infarct area, requires a craniotomy, and also a fairly high degree of neurosurgical skill (Carmichael 2005).

Photothrombosis is induced by the intravenous injection of a photosensitive dye (ie. Rose Bengal or erythrosin B) followed by the irradiation of the exposed skull with a specific wavelength (Watson et al., 1985). This generates oxygen radicals, causing endothelial damage and a prothrombotic state and secondary ischemia (Dietrich et al., 1986; Ginsberg & Busto, 1989). This method requires the targeted vessels to be close to the surface of the cortex and near the skull in order for the irradiation to reach them. It is therefore primarily limited to producing cortical stroke. While this method allows for a very specific and small area of ischemia, it also has a secondary effect of increased cerebral edema. Unlike the pathophysiology in human stroke, there is a very small area of penumbra around the infarct as there is very little collateral flow leading to reperfusion due to the microvascular insult occurring within every exposed vessel (Carmichael 2005).

Lastly, the ET-1 method of inducing ischemia requires the application of ET-1 at predetermined coordinates to induce a localized ischemic injury. ET-1 is a potent vasoconstrictor



that reduces cerebral blood flow in a rapid but not immediate manner, and induces ischemia with subsequent gradual reperfusion over several hours (Yanagisawa et al., 1988; Carmichael 2005; Windle et al., 2006; Durukan & Tattisumak, 2007; Macrae, 2011). This approach can either utilize a more invasive model, with direct visualization of the area of interest (via craniotomy and topical application), or a less invasive model, with microneedle injection using pre-determined coordinates and stereotactic measurements from skull or brain surface through small burr holes. I chose to use the stereotactic method, as it induces a more reproducible and persistent ischemia than topical application (Sharkey et al., 1993). Good intra-operative and post-operative care is necessary, but less specialized surgical skill is required, making it less surgically demanding (Macrae, 2011). We have used the ET-1 model with recurrent success in our lab, targeting various regions of the rat brain (Biernaskie & Corbett, 2001; Windle et al., 2006; Clark et al., 2009; Ploughman et al., 2009; Hewlett et al., 2014). I therefore opted to proceed with the use of the ET-1 model of ischemia for this project.

## **1.2 Biological Mechanisms Underlying Post-Stroke Recovery**

As was recently reviewed by Carmichael (2016), cortical reorganization and repair following stroke involves a number of biological mechanisms within the cortex adjacent to the infarcted region (the peri-infarct cortex) as well as the contralateral cortex. This includes axonal sprouting to regions of the midbrain, striatum and the spinal cord. This neuroplasticity is achieved through generation of new neurons, oligodendrocytes and novel connections, and in animal models has been shown to correlate with an improvement in function and increased post-stroke performance. Typically, axonal sprouting is limited due to the expression of glial growth inhibitory molecules, yet these glial growth inhibitors are down regulated following an ischemic

event (Li & Carmichael, 2006) and if blocked pharmacologically axonal sprouting is subsequently enhanced in the motor, premotor, and somatosensory regions (Overman et al., 2012). Functional human imaging studies (Grefkes & Fink, 2014) have also shown similar connections and cortical remapping following stroke in the same regions demonstrated in rodents and non-human primates (Dancause et al., 2005).

There are a number of similarities between post-stroke recovery of function and the process of motor learning, and studies in non-injured animals illustrate similar mechanisms including long-lasting alterations in dendritic spine turnover (Silasi & Murphy, 2014; Carmichael 2016). Studies have demonstrated that inhibiting or blocking GABA receptor signaling promotes neuronal excitability and improves learning and memory in rodent models (Glykys & Mody 2007). In response to stroke, however, the peri-infarct cortex exhibits increased GABA signaling which produces a hypoexcitable state which is thought to represent a neuroprotective response to limit injury (Carmichael 2016). However, this response may interfere with post-stroke recovery. If this inhibitory signal is blocked through pharmacological manipulation, post-stroke recovery of function is enhanced (Clarkson et al., 2010; Lake et al., 2015).

Depending on the size of the initial infarct, the underlying mechanisms and capacity for repair and recovery differ. If the injured area is small, recovery of function occurs through the spared peri-infarct cortex, as the function and connectivity of this region is very similar to the infarcted area. If the region is larger and involves the motor cortex, however, more distally located cortical tissue accomplishing a similar function may be recruited including the premotor area (Zeiler et al., 2013) or the contralateral motor cortex (Biernaskie et al., 2005). In both scenarios, animal studies have demonstrated an improvement in function which correlates with

the degree of increased connectivity between adjacent and more distal structures. There is also, however, a well-established effect of timing, with significant negative outcomes associated with additional ‘stress’ exposure during the first few days after stroke. If GABA signaling is inhibited within the first 3-5 days following stroke in a rodent model, instead of improving functional outcomes and recovery, the opposite occurs and infarct size is actually increased (Clarkson et al., 2010). Similar clinical findings have been observed in human studies (Dromerick et al., 2009; AVERT Trial Collaboration Group, 2015), with intensive rehabilitation introduced during the first week following stroke causing an impairment in post-stroke recovery when compared to more delayed rehabilitation. Again, this provides additional support for an ‘optimal window’ of timing for post-stroke rehabilitation while also emphasizing the importance of well-researched animal models and their application to human subjects.

### **1.3 Recruitment of Peri-Infarct and More Distal Neural Circuitry in Post-Stroke Recovery**

Many studies, both animal and human, have demonstrated that most post-stroke recovery occurs within the first 3 months following stroke. As previously described, the peri-infarct cortex has been shown to reorganize soon after stroke, and works to re-establish function in animal models through a variety of changes (Castro-Alamancos & Borrel, 1995; Brown 2006; Dijkhuizen et al., 2003; Winship & Murphy, 2009). These changes include sprouting, reorganization of cortical maps, and formation of new dendritic spines in the peri-infarct region (Brown et al., 2008; Murphy & Corbett, 2009; Zhang et al, 2005; Carmichael 2016).

Neuroplasticity has been studied for decades, with a large body of literature describing and reviewing the mechanisms contributing to post-stroke recovery (Zeiler & Krakauer, 2013; Silasi & Murphy, 2014; Carmichael 2016; Dancause & Nudo 2011). As previously mentioned,

the injured brain has a number of adaptations which contribute to recovery of function following ischemia, and the most relevant to this thesis is the reorganization of cortical maps to support the uninjured and peri-infarct cortex. Most commonly, the more frontal regions of the brain appear to contribute to the performance of complex behaviours in both animal and human studies.

A study by Zeiler and colleagues (Zeiler et al., 2013) demonstrated reinstatement of reaching deficits in mice that had recovered from caudal forelimb area cortical strokes when a second stroke affecting the medial premotor cortex was induced. They also showed that the initial recovery correlated with a reduction in inhibitory interneuron markers in this premotor region, similar to the studies previously discussed (Clarkson et al., 2010; Lake et al., 2015). This study and others described above suggest that depending on the location and size of stroke injury, more remote areas of the stroke connectome outside of the peri-infarct cortex can contribute to recovery (Silasi & Murphy, 2014; Brown et al., 2009). One such remote region is the medial prefrontal cortex (mPFC) which is thought to have analogous function to the human dorsolateral prefrontal cortex (Ongur & Price, 2000).

As discussed in the review by Kolb and colleagues (2012), the prefrontal cortex (PFC) receives its principal input from the mediodorsal nucleus of the thalamus, and is composed of a group of related regions, as opposed to an anatomically distinct region. The function of the PFC is largely to organize behaviour in a temporal manner, as it supports cognitive function by integrating input from the environment and learned experiences. Thus its primary function is to organize behaviour both within time and within context, suggesting it plays an important supportive role in coordinating planned and learned motor tasks, especially in aging or if the motor cortex has suffered an ischemic injury. The scaffolding theory of aging and cognition and

the recruitment of additional circuitry may therefore play an important role in neuroplasticity and recovery following ischemic events (Park & Bischof, 2013).

#### **1.4 Post-Stroke Recovery: Motor Learning, Aging and the Medial Prefrontal Cortex**

In an fMRI study by Heuninckx and colleagues (2008), it was reported that older subjects exhibited more extensive brain activation (ie. in the prefrontal cortex) than younger controls while performing a motor coordination task. Importantly, this increased activation was positively correlated with performance in the elderly, suggesting that this additional activation (termed *compensatory recruitment*) was in fact performance enhancing. This is particularly interesting because the prefrontal cortex is an area of the brain typically not involved in motor function (as was observed in the younger group of subjects), yet was shown to be recruited in a beneficial manner in the older population. Similar research findings have also been reported in other studies demonstrating compensatory neural activation in older adults (Park & Bischof, 2013). This has since been used to develop the scaffolding theory of aging and cognition, which is a theoretical model that suggests the recruitment of additional circuitry (ie. the prefrontal cortex) improves declining brain function, and those who can accomplish this more effectively are more successful than cohorts who do not display this same compensation. It continues to remain an active point of discussion as to whether the basis behind neuroplasticity in the aging brain is a result of *reserve* and an increased supply of available neural resources secondary to experiences, or whether it is neural compensation and the ability to draw more efficiently from pre-existing neural networks (Park & Bischof, 2013).

This has interesting implications for stroke research as the findings suggest that like aging, motor relearning and compensation may be contributing to post-stroke recovery. The

existence of compensatory recruitment holds promise as a novel method of improving post-stroke recovery through the strengthening of complimentary pathways. Following a motor stroke for instance, where the motor cortex suffers irreparable damage, a potential strategy of enhancing post-stroke recovery of function may be to strengthen complimentary pathways involving these prefrontal connections. This idea is suggested by studies showing contribution of distal brain regions and connections as alternative substrates for enhancing neuroplasticity and recovery (Brown et al., 2009; Biernaskie et al., 2005; Zeiler et al., 2013, Zeiler & Krakauer, 2013; Silasi & Murphy, 2014).

In view of the above discussion, the goal of this thesis was to examine the effect of antecedent mPFC damage on post-stroke recovery in a rodent model of motor stroke. This stroke model serves to increase our knowledge and understanding of the injured brain, by allowing us to gain a better understanding of the role, if any, that the mPFC plays in the initial relearning following a well characterized rat motor stroke model.

As was previously discussed in the section titled ‘Animal Models of Stroke’, there are a number of methods by which to induce ischemia for a rodent model of stroke, yet the methods of induction of mPFC ischemia are less numerous than those that target the territory of the MCA, for a number of practical reasons. The mPFC does not lend itself to mechanical obstruction via suture or monofilament, as the anterior cerebral artery which supplies the mPFC is not readily accessible. Additionally, photothrombosis is not suitable, as the mPFC is a deeper cortical structure and would not allow the irradiation to reach a more ventral target. The decision was therefore made to use ET-1 for both ischemic injury models, to target both the territory of the MCA and the mPFC.

Based on previous research (Heuninckx et al., 2008), I hypothesized that there would be less evident motor recovery following MCAo in rats that had previously experienced mPFC injury compared to rats with the same MCAo stroke but an intact mPFC. I also hypothesized that this difference would be more pronounced in the middle-aged group, reflecting a difference in neuroplasticity and compensation in the aged brain, with the middle-aged rats showing less motor recovery than the younger cohort.

This study design also allows me to observe differences in recovery of function over time following a motor stroke in a previously injured brain. By assessing these animals at multiple time points, I can compare the degree of spontaneous recovery in animals with damage to the mPFC to those without. This will provide additional information about the extent to which this region of the rodent brain is involved in recruitment of additional, supportive neural networks and its subsequent impact on neuroplasticity in the recovering brain.

Through the work of Heuninckx and colleagues (2008) among others, I hypothesized that the mPFC plays a role in recovery of motor performance in an age-dependent fashion. This additional recruitment occurs in the aged human brain in healthy, well-performing adults and appears to be beneficial to motor performance. It is my hypothesis that similar recruitment also occurs in the rodent brain in an age-dependent manner. Accordingly, I predict that prior mPFC ischemia will result in more substantial impairments in recovery following MCAo in aged animals compared to younger rats.

## **2. Methods**

### **2.1 Subjects**

A total of 128 male Sprague-Dawley rats (Charles River, Montreal, Canada) were used in this study. Age equivalent animals were pair-housed and maintained on a reverse 12:12h light-dark cycle (lights on at 1900h) with *ad libitum* access to food and water unless otherwise indicated. Behavioural testing was conducted during the dark cycle. All rats received daily handling to allow them to acclimatize to the environment for at least 9 days prior to initiating experimentation. The aged animals arrived at approximately 6 months of age, and were permitted to ‘age’ in the same conditions, with daily handling, for an additional 6 months. All procedures were approved by the Memorial University Animal Care Committee and conformed to the Canadian Council on Animal Care guidelines.

### **2.2 Experiment 1**

#### **2.2.1 Study Design**

The aim of this initial experiment was to evaluate the effects of medial prefrontal cortex (mPFC) ischemia on motor and cognitive behaviour. An animal model of mPFC ischemia as it relates to motor function has not been previously characterized, therefore prior to initiation of the main Experiment (Experiment 2), work was initiated to ensure that injury to the mPFC alone does not result in generalized motor or global cognitive deficits. All animals were exposed to the same training and testing paradigms (Figure 1a).

Twenty-five ‘young’ (~3 mo) and 16 ‘middle-aged’ (~12 mo) rats weighing approximately 350 and 675 g, respectively, at the time of surgery were used in Experiment 1. With respect to the young animals, there were three experimental conditions: **1** - bilateral mPFC



ischemia ( $N = 8$ ); **2** - unilateral (left sided) mPFC ischemia ( $N = 8$ ); and **3** - a control group receiving sham surgery (burr holes alone) ( $N = 9$ ). Surgical mortality led to the use of twenty-four young rats in the final analysis ( $N = 8$  in each group). With respect to the middle-aged animals, there were two experimental conditions: **1** - bilateral mPFC ischemia ( $N = 8$ ) and **2** - control group, receiving sham surgery (burr holes alone) ( $N = 8$ ). Subsequent difficulties with training necessitated a change in the final numbers included in each group for the staircase and radial arm maze testing. Please see below for details, described within the relevant section.

## 2.2.2 Surgery

### 2.2.2.1 Medial Prefrontal Cortex Ischemia

Endothelin-1 (ET-1, 400 pmol/ $\mu$ l in sterile H<sub>2</sub>O), a vasoconstrictive peptide, was injected into the medial prefrontal cortex to induce localized ischemic injury. Animals were randomly assigned to receive bilateral (four injections; 2/hemisphere), unilateral (left sided; two injections) mPFC ischemia or control surgery where burr holes were drilled at the same coordinates but without lowering the needle into the brain. Briefly, animals were anesthetized with Isoflurane (4% induction, 2% maintenance) in a 30:70 O<sub>2</sub>/N<sub>2</sub>O mixture and rectal temperature was maintained at  $\sim 37.0^{\circ}\text{C}$  for the duration of the surgery by a self-regulating heating blanket (Harvard Apparatus, Holliston, MA, USA). The injection coordinates, relative to bregma, were as follows:

- 1) Anteroposterior (AP), +4.0 mm; mediolateral (ML),  $\pm 0.7$  mm; dorsoventral (DV), -4.0 mm
- 2) AP +3.0 mm; ML  $\pm 0.7$  mm; DV -3.7 mm

The dorsoventral depth was measured from skull surface, and a beveled syringe was used to deliver ET-1, with the bevel facing anterior. Once the needle was inserted and reached the appropriate depth it was left in place for 1 min before the injection of ET-1. Endothelin-1 was then injected (0.8  $\mu$ l) over 5 min and the needle was left undisturbed for 2 min following the injection to minimize both overflow into the contralateral hemisphere and backflow into adjacent brain regions. The entire surgical procedure lasted approximately 30, 60, or 90 min (for the control, unilateral, and bilateral operations, respectively). Following recovery the rats were returned to paired-housing (with their original cage mate) and were handled daily for a week to monitor weight gain and recovery.

### **2.2.3 Behavioural Assessments**

#### 2.2.3.1 Motor Performance

Unilateral weakness or hemiparesis affecting the contralateral side to the ischemic infarct is the most common impairment following a stroke. Over 50% of stroke survivors experience significant residual deficits in motor function (Mayo et al, 2002; Duncan et al, 1992), preventing their independence in activities of daily living, and leading to a persistent effect on quality of life. As such, it is important to make use of sensitive tests of motor function in assessing post-stroke performance, to accurately reflect and model the impact of stroke in the human population.

##### *2.2.3.1.1 Staircase Test of Fine Motor Function*

The staircase test was designed by Montoya et al. (1991) to assess skilled reaching performance in the rodent population, and consists of a Plexiglas® box with a removable baited

double staircase (one for each forelimb). The rat enters the box, with its ventral surface resting on a raised platform support, and both forelimbs free to reach for food pellets placed on gradually distal levels that resemble a staircase. There are three food pellets placed on each level of the staircase, and 7 levels in total, for a total of 21 available pellets. Those placed on the stair closest to the head of the rat are the most accessible, and those more distal are progressively more difficult.

Similarly to humans, if given the option, rats will compensate for impaired motor functioning in one paw by using their opposite and unimpaired limb whenever possible. This staircase apparatus by Montoya and colleagues prevents the subjects from using their unimpaired paw, as the staircases for each side are separated and cannot be reached by the contralateral limb. This allows for an objective measurement of post-stroke impairment by comparing pre and post-stroke performance for the contralateral limb, and also allows for a sensitive measure of persistent deficits over time.

This apparatus requires extensive training (approximately 2 x 15 minute trials per day for 10-14 days), but with time each subject learns to successfully and persistently reach an average of 17-19 pellets per trial. This allows for significant impairments to be objectively noted in the post-stroke period, and a number of studies have demonstrated that staircase performance deficits persist for weeks following stroke (Biernaskie & Corbett, 2001; Clarke et al. 2009; Ploughman et al. 2007), supporting the sensitivity of this testing paradigm for detecting post-stroke impairments that mimic the persistent deficits in human stroke survivors.

#### *2.2.3.1.1 Staircase Assessment*

For the purposes of this study and as per previously described protocols, all animals were mildly food restricted (~90-95% of free feeding body weight), and trained in the Montoya

staircase reaching task for 2 weeks prior to surgery (Montoya et al., 1991). Training consisted of two 15 min trials per day, to reach for 45 mg food pellets (TestDiet, Richmond, IN, USA). The staircase apparatus had 7 steps/side that each held 3 food pellets, all situated at a progressively more difficult distance from the animal. Training was continued until the rat successfully obtained a pre-determined criteria of at least 12 out of the possible 21 pellets (although, on average animals reached to a total of 17 pellets), and a standard deviation  $\leq 2$  pellets over a period of eight trials. For experiment 1, 4 young animals were not successfully trained to criteria for inclusion in staircase testing, as well as 8 middle-aged subjects. This resulted in the following groups for motor assessment for the youngest animals: **1** - bilateral mPFC ischemia ( $N = 8$ ); **2** - unilateral (left sided) mPFC ischemia ( $N = 8$ ); and **3** - sham surgery ( $N = 4$ ). Due to low training success in the middle-aged animals (8 rats did not meet inclusion criteria), there was an insufficient number of animals to appropriately power two groups. This resulted in only one group being included in the staircase assessment: bilateral mPFC ischemia ( $N = 8$ ), and a control group was therefore not included.

The number of pellets consumed during the last four trials (prior to surgery) was averaged for every rat and this was a measure of baseline proficiency. The unilateral (left hemisphere) young ischemic animals were all successfully trained using their right paw in order to assess any potential motor effects of the mPFC ischemia. Those animals receiving either bilateral or sham surgeries were assessed using their most successful paw at baseline. On post-stroke day 5 and 6, every rat was given four 15 min test trials (2 per day, following the same procedure as before) and the average number of pellets consumed was compared to each rat's baseline performance.

### 2.2.3.2 Cognitive Performance

As was previously described, cognitive deficits following ischemic events are particularly debilitating. Damage to the prefrontal cortex especially can result in deficits in executive function including difficulty with planning, behavioural flexibility, working memory, temporal organization of memory, and attention set shifting (Cordova et al., 2014; Livingston-Thomas et al., 2015; Deziel et al., 2015). In light of this, it is important to employ a sensitive model of cognitive performance that can detect significant deficits that may impair rodents' ability to perform motor tasks, as this is the primary research question of this study.

#### *2.2.3.2.1 Radial Arm Maze Test of Working Memory*

The radial arm maze (RAM) was originally designed to test rodent performance in a test of working and reference memory (Olton and Samuelson, 1976). It consists of an elevated central platform from which eight equidistant arms (or runways) radiate, each with Plexiglas® walls to prevent inter-arm traverses. Each arm has a small sunken cup at the far end to keep a food pellet hidden from sight, and which serves as the motivation to navigate the maze. Importantly, all of the arms are identical, and the maze remains in the same orientation within the test room.

This maze uses the rodent's natural foraging abilities and motivation in order to assess short-term memory. As in their natural habitat, the subjects are rewarded for entering a novel region of the environment (a new arm), and receive no reward for entering a previously visited arm from which the food source has already been consumed. As the rats learn to navigate the maze, they make use of both intramaze (scents) and extramaze (visual) cues in order to recognize novel arms and avoid reentry errors (a working memory error). Over consecutive days, the

subjects learn to avoid the arms that they have already explored, and to enter only novel arms in order to successfully navigate the maze. Each new day is begun with all eight arms baited, and the subjects do not need to remember the previous day's choices. This functions to assess one aspect of cognitive function through short-term (working) memory. Reference memory, assessed using the 4-arms baited configuration of the RAM, was not tested in this study.

#### *2.2.3.2.1.1 Radial Arm Maze Assessment*

Thirty-six animals were included for RAM cognitive assessment, in 5 total groups. As previously described at the beginning of the description for Experiment 1, this included 3 groups of young rats: bilateral mPFC ischemia ( $N = 7$ ); unilateral mPFC ischemia ( $N = 7$ ); sham procedure ( $N = 8$ ), and 2 groups of middle-aged rats: bilateral mPFC ischemia ( $N = 7$ ); sham procedure ( $N = 7$ ). All animals were food restricted (to ~85-90% of free feeding body weight) and assessed using the 8-arms baited radial arm maze configuration (RAM; 70 cm arms; 12 cm wide; 35 cm centre platform; 20 cm clear Plexiglas® walls) (Olton and Samuelson, 1976). Animals were assessed using the RAM protocol previously described (Hartman *et al.*, 2005; Langdon *et al.*, 2008), beginning on post-stroke day 7. Briefly, animals received 2 consecutive days (5 min/trial) of acclimatization to the maze, where 4 pellets were placed along the length of each arm, encouraging exploration. On the third day, to begin testing, pellets were restricted to the food cups at the end of each arm. Animals were tested once per day on consecutive days, and the latency to obtain all rewards (to a maximum of 5 minutes), as well as the number of re-entry (working memory) errors were recorded. Testing continued until every subject achieved the criteria of <2 errors per trial on 4 consecutive days, or until 4 weeks had passed. The number of

trials to reach criteria was recorded for each subject. In each of the 5 groups there was 1 animal that did not reach criterion within the 4 allotted weeks.

#### **2.2.4 Histology**

Rats were sacrificed over a series of two days (due to time constraints), beginning the day after testing was completed. All animals in Experiment 1 were evenly divided into two groups and euthanized during two days following the date the last animal met criteria for RAM assessment. Animals were sacrificed by transcardial perfusion using ice cold heparinized saline followed by 10% buffered formalin. Animals were decapitated and heads immersed in 10% buffered formalin for 4 hours at 4°C. The brains were then removed from the skull and immersed in a 20% sucrose solution in phosphate buffered saline (PBS) at 4°C until saturated, then frozen by gradual submersion in cold isopentane kept on dry ice, and then stored in a freezer at -20°C until sectioning. Coronal sections were cut using a cryostat at a thickness of 40 µm and stained with hematoxylin and eosin (H&E) to assess infarct area.

#### **2.2.5 Infarct Volume Measurements**

Infarct volumes were measured by an observer blinded to experimental condition and group assignment. Measurements were taken from the H&E stained sections using NIH Image Software (ImageJ 1.36b software for Mac, downloaded from the public domain, National Institutes of Health, USA, <https://imagej.nih.gov/ij/>). Every 18<sup>th</sup> section was analyzed, beginning at the section in which ischemic damage was first visualized, and proceeding until the end of the lesion. The infarct volume for each section was calculated using the following formula: Area of intact tissue in the contralateral hemisphere – Area of intact tissue in the ipsilateral (ischemic)

hemisphere. In animals that underwent bilateral injury, the intact hemisphere was estimated using the intact tissue immediately adjacent to the infarcted area and bridging the infarct as though no injury had occurred. Infarct volume was calculated by accounting for the number of sections (including the 17 discarded sections) as well as their thickness (40  $\mu\text{m}$ ), in order to calculate a volume for total hemispheric infarct. Infarct volumes were then compared between groups and ages for statistical analysis, as will be described in the following section, and presented as a measure of volume in  $\text{mm}^3$ .

No animals were excluded based on volume of ischemic injury being too large or too small.

#### **2.2.6 Statistical Analyses**

All statistical tests were run as described, using either univariate analysis or ANOVA, as appropriate. If a post-hoc analysis was required and the assumption of homogeneity of variance was satisfied, the post hoc test Tukey honestly significant difference (HSD) test was run. This was applicable to all situations where a statistically significant difference was found, most notably in the assessment of infarct volumes.

Statistical analysis for staircase performance in the younger group was done using repeated measures ANOVA. All three groups were determined to be equal in performance across groups prior to mPFC ischemia using univariate analysis. Staircase performance in the middle-aged group following bilateral mPFC ischemia was assessed using a paired t-test, comparing post-stroke day 5 to pre-stroke performance. This was required because of the unfortunately low staircase-training rate in the middle-aged group that significantly decreased the total number of subjects in this age group. The decision was therefore made to use all available animals for



mPFC ischemia to assess motor performance, using a t-test to compare pre and post-stroke performance in this group. The radial arm maze, as previously discussed, was assessed using number of days to criteria and was analyzed using a one-way ANOVA for both the young group and the middle-aged group. Univariate analysis was used to assess the effect of age and bilateral stroke on staircase performance between the young and middle-aged groups. Significance level was set at  $p < .05$ , and all values are represented as mean  $\pm$  standard error of the mean.

For comparison of infarct volume in each hemisphere within each group (young group with unilateral mPFC ischemia, young with bilateral mPFC ischemia, and middle-aged with bilateral mPFC ischemia), a paired samples t-test was used. Following this assessment, an ANOVA was run to compare total infarct volume between groups. As this was found to be significant, a post hoc Tukey HSD test was used. As described, infarct volume was calculated for total hemispheric infarct as compared to the intact hemisphere and compared between groups.

## **2.3 Experiment 2**

### **2.3.1 Study Design**

The aim of Experiment 2 was to compare the effects of MCAo ischemia with and without an additional mPFC ischemic injury in the same 2 cohorts of animals: young (~ 3 mo) and middle-aged (~12 mo) rats.

Thirty-four ‘young’ (~350 g) and 33 ‘middle-aged’ (~675 g) Sprague-Dawley rats were included in Experiment 2. Animals were pair-housed with another animal of the same age and maintained on a reverse 12:12h light-dark cycle (lights on at 1900h) with *ad libitum* access to food and water unless otherwise indicated. All behavioural assessments and protocols were

conducted as in Experiment 1, with all animals in Experiment 2 exposed to the same training and testing paradigms (Figure 1b)

Within each age cohort animals were randomized into one of two conditions: 1) ET-1-induced MCAo combined with bilateral mPFC ischemia: young (n=17) and middle-aged (n=17) and 2) ET-1-induced MCAo without mPFC ischemia: young (n=16) and middle-aged (n=15). One animal from the youngest group and one from the middle-aged group died prior to study completion.

## **2.3.2 Surgery**

### *2.3.2.1 Medial Prefrontal Cortex*

Bilateral mPFC was induced as in Experiment 1 using similar coordinates:

- 1) AP +4.0 mm; ML  $\pm$  0.7 mm; DV -4.0 mm
- 2) AP +3.0 mm; ML  $\pm$  0.7 mm; DV -3.7 mm

Animals undergoing sham mPFC surgeries received four burr holes (2/hemisphere) and as in Experiment 1 were not exposed to needle injury.

### *2.3.2.2 Middle Cerebral Artery Occlusion*

Two weeks following mPFC ischemic or sham surgery, all animals underwent a second surgery to induce ischemia via middle cerebral artery occlusion (MCAo), again with a single infusion of ET-1 (400 pmol/ $\mu$ l in sterile H<sub>2</sub>O). The surgical procedure was similar to that previously described for anesthesia, temperature regulation and induction of ischemia, with a difference in coordinates as follows:

Young (aged 3 months) rats:

AP +0.9 mm; ML  $\pm$  5.2 mm; DV -9.1 mm

Middle (aged 12 months) rats:

AP +0.9 mm; ML  $\pm$  5.2 mm; DV -9.4 mm

Measurements were taken relative to bregma, the dorsoventral depth was measured from skull surface at the site of the burr hole, and a beveled syringe was used to deliver ET-1 with the bevel facing anterior. All animals received either right or left sided MCAo ischemia based on their performance in staircase training, receiving the injection of ET-1 in the hemisphere opposite the paw showing the greatest number of reaches in the staircase. Once the needle tip reached the appropriate depth it was left in place for 1 min before the injection of ET-1. Endothelin-1 was then injected (1  $\mu$ l) over 2 min and the needle was left undisturbed for 2 min, followed by two more infusions carried out in the same manner for a total of 3  $\mu$ l of ET-1. Following the last infusion of ET-1, with the needle having been in place for a total of 11 minutes, the needle remained undisturbed for an additional 3 minutes before being removed very slowly and carefully. This was to minimize overflow and backflow of ET-1 into adjacent brain regions. The entire surgical procedure lasted approximately 45 min. Following recovery, the rats were returned to paired-housing (with their original cage mate) and were handled daily for a week to monitor weight gain and recovery.

### **2.3.3 Behavioural Assessment**

#### *2.3.3.1 Staircase Testing*

Animals were trained as was previously explained in Experiment 1. Surgical and control groups were randomly assigned based on pre-stroke (baseline) staircase performance to ensure there was no difference between groups at the onset of the study.

On MCAo post-stroke day 5 and 6, every rat was given four 15-minute test trials (2 per day, following the same procedure as training) and the average number of pellets consumed was compared to each rat's baseline performance. This testing was repeated again on post-stroke week 2, week 4, and week 6 in order to assess performance over time.

#### **2.3.4 Histology**

The same method of sacrificing and preparing brains was followed for Experiment 2 as was previously described for Experiment 1. Animals were euthanized over a series of 2 days (due to time constraints), beginning the day immediately following completion of behavioural assessments.

#### **2.3.5 Infarct Volume Measurements**

Infarct volumes were measured by an observer blinded to experimental condition. Measurements were taken from the H&E stained sections using NIH Image Software (ImageJ 1.36b software for Mac, downloaded from the public domain, National Institutes of Health, USA). Every 18<sup>th</sup> section was analyzed, beginning at the section in which ischemic damage was first visualized, and proceeding until the end of the lesion, for both the mPFC and MCA regions. The infarct volume for each section was calculated using the following formula: Area of intact tissue in the contralateral hemisphere – Area of intact tissue in the ipsilateral (ischemic) hemisphere. In animals that underwent bilateral mPFC injury, the intact hemisphere was estimated using the intact tissue immediately adjacent to the infarcted area and bridging the infarct as though no injury had occurred. Infarct volume was calculated by accounting for the number of sections (including the 17 discarded sections) as well as their thickness (40  $\mu$ m), in order to calculate a volume for total hemispheric infarct. The regions were then separated into

cortical ischemic volume or striatal ischemic volume. This was then presented as a measure of volume in  $\text{mm}^3$ , and compared between groups and ages, as will be described in the following section.

No animals were excluded based on volume of ischemic injury being too large or too small.

### **2.3.6 Statistical Analyses**

Statistical analysis for staircase performance in experiment 2 was again done using repeated measures ANOVA. All three age groups were determined to be equal in performance across groups prior to mPFC ischemia using univariate analysis. Staircase performance following bilateral mPFC ischemia was assessed using repeated measures ANOVA, comparing within-subjects' performance across all four time points.

Infarct volume was compared using univariate analysis as a one-way ANOVA. As described, infarct volume was defined as cortical or striatal, which was combined to present as total infarct volume.

Significance level was set at  $p < .05$ , and all values are represented as mean  $\pm$  standard error of the mean.

## **3. Results**

### **3.1 Experiment 1**

#### *3.1.1 Motor Function – Staircase Test of Fine Motor Function*

The first objective was to rule out any difference in motor function following bilateral or unilateral mPFC ischemia in the young group. There was no difference between groups when comparing pre or post-ischemia performance in the staircase test among the young group, with

the means ranging only from 17.1 to 18.5 pellets across the three groups ( $\pm 1.02$  to  $\pm 1.73$  SEM, Figure 2a). There were no significant impairments in staircase performance resulting from either unilateral or bilateral mPFC ischemia both relative to either pre-stroke performance, or between groups (time x group,  $p = .655$ ; group,  $p = .289$ ; time,  $p = .171$ ) (Figure 2a).

The second objective was to determine whether there was any difference in the middle-aged group following bilateral mPFC ischemia as compared to baseline. The result was similar to that observed in the young rats, with no significant impairments observed relative to baseline in the middle-aged rats following bilateral mPFC ischemia (time x group,  $p = .829$ ; time,  $p = .616$ ). There was, however, significant impairments in staircase performance due to age at both time point (group,  $p < .001$ ), indicating that older animals are worse at performing the task overall (Figure 2b). The differences in average number of pellets reached between age groups was significant, with the middle-aged group reaching an average of 15.3 pellets ( $\pm 1.83$  SEM) at baseline compared to the young group which reached an average of 18.3 pellets ( $\pm 1.37$  SEM) at baseline. This difference between age groups also remained significant following mPFC ischemia, with the middle-aged group reaching an average of 14.9 pellets ( $\pm 1.06$  SEM), and the young group reaching an average of 18.2 pellets ( $\pm 1.02$  SEM) (Figure 2b). There was also a difference in successful training between both age groups, as only 4 (of a possible 24) young animals failed to meet staircase training criteria prior to surgery, as compared to 8 (of a possible 16) middle-aged animals which were not successfully trained in staircase (83% vs 50% successful training rate).

### *3.1.2 Cognitive Function – Radial Arm Maze*

All available animals were assessed using the RAM, yet in each of the 5 groups there was 1 animal that did not reach criterion within the 4 allotted weeks. Final statistical analysis was

therefore done using a total of 22 young animals and 14 middle-aged animals. This included bilateral mPFC ischemia ( $N = 7$ ), unilateral mPFC ischemia ( $N = 7$ ), and sham procedure ( $N = 8$ ) in the young group, and bilateral mPFC ischemia ( $N = 7$ ) and sham procedure ( $N = 7$ ) in the middle-aged group.

Medial PFC ischemia did not disrupt cognition as assessed in the radial arm maze (number of days to criterion). There were no significant impairments in radial arm maze performance for either unilateral or bilaterally injured young rats relative to sham ( $F_{2,19} = 0.761$ ,  $p = .481$ ), with all groups requiring very similar number of days to meet criterion (Figure 3a).

This same result was replicated when comparing both age groups, as neither young nor middle-aged rats exhibited cognitive impairments as a result of mPFC injury relative to sham (age x stroke,  $p = .853$ ; stroke,  $p = .970$ ). Similar to the staircase task of motor performance, middle-aged rats were again more significantly impaired in their ability to learn the task as compared to young rats, requiring significantly more days to meet criterion as compared to the younger cohort (age,  $p < .001$ ) (Figure 3b).

### 3.1.3 Infarct Volume

There were no significant differences between damage in the left and right hemispheres for either the young or middle-aged bilaterally injured groups. Importantly, the young unilaterally injured group had no damage in the contralesional hemisphere, suggesting that the stroke model used to induce ischemic damage in this study was precise, did not cause collateral damage to adjacent areas of the cortex, and gave similar volumes of injury on both sides (Figure 4a).

As a total measure of infarct volume, there was a significant difference between groups ( $F_{2,21} = 3.831, p = 0.038$ ). Using the Tukey HSD post hoc comparison, a significant difference was found between infarct volume of the young group following unilateral ischemia as compared to the middle-aged group following bilateral ischemia ( $p = .03$ ). There was, however, no significant difference in infarct volume detected between the young bilaterally injured and the middle-aged bilaterally injured groups (Figure 4b). This suggests that this bilateral stroke model induces similar infarct volumes regardless of age, and the subsequent cognitive and motor performance scores can be compared between age groups as having had a similar degree of cerebral injury.

#### *3.1.4 Overall Result of Experiment 1*

Bilateral mPFC ischemia did not alter performance in the staircase test or the radial arm maze test when comparing baseline performance to post-ischemia performance. These results demonstrate that mPFC stroke does not cause skilled reaching impairments or impairments in radial arm maze tests in young *or* middle-aged rats.

Experiment 1 did, however, illustrate that there is a significant difference in the performance of middle-aged animals in both the staircase test and the RAM as compared to the younger cohort. This was demonstrated in their training success and performance at baseline, and remained constant in their post-stroke performance as well.



## **3.2 Experiment 2**

### *3.2.1 Motor Function – Staircase Test of Fine Motor Function*

As opposed to what was observed in experiment 1, there was no difference in baseline staircase performance among groups prior to surgery. There were no differences in baseline performance between groups due to age ( $F_{2,42} = 0.410, p = 0.596$ ), and no difference between those assigned to the control group or to the mPFC ischemia group ( $F_{1,42} = 0.286, p = 0.666$ ). The average number of pellets reached in each group at baseline varied from 17.3 to 17.8 ( $\pm 1.04$  to  $\pm 1.73$  SEM, data not illustrated).

With respect to post-stroke staircase performance, there was a significant effect of both time ( $F_{3,126} = 26.268, p < 0.001$ ) and of age ( $F_{2,42} = 4.715, p = 0.014$ ), but no effect of mPFC ischemia ( $F_{1,42} = 0.115, p = 0.736$ ). There were no significant interactions of time and mPFC ischemia ( $p = 0.204$ ), of time by age ( $p = 0.713$ ), or of mPFC ischemia and age ( $p = 0.713$ ). There was also no significant three-way interaction of time, mPFC ischemia, and age ( $p = 0.370$ ) (Figure 5).

Additionally, a significant effect of time was observed, with an improvement in staircase performance at all time points ( $p < 0.001$ ) following surgical intervention, as all groups increased their performance across weeks by the same degree (Figure 5).

### *3.2.2 Infarct Volume*

There was no significant difference between any group with respect to total infarct volume (Figure 6).

Upon further investigation of striatal infarct volumes, there was no significant stroke by age interaction observed, as there was again no difference between groups with respect to striatal

infarct volumes (Figure 7a). There was, however, a significant effect of age, as middle-aged animals had significantly smaller striatal infarct volumes than young animals ( $p = .006$ ), regardless of the presence of a prior mPFC injury (Figure 7b).

## **4. Discussion**

### **4.1 Study Goal**

The goal of this study was to determine if prior damage to the mPFC impaired recovery following a subsequent motor stroke. As such, this represents a novel animal model of recurrent stroke; a common clinical problem. By comparing the motor effects of a single motor stroke to the effects of a motor stroke following a previous mPFC stroke, I sought to compare the role of compensatory recruitment, or reserve, and its role in immediate post-stroke motor performance. Lastly, I sought to compare the effects of this ischemic model across 2 different age groups to assess any differences in motor performance and the impact of MCAo and mPFC ischemia in young and middle-aged rats. The mPFC was chosen for a number of reasons. As discussed in the introduction, it arose from a study published by Heuninckx and colleagues (2008) and a number of others, using both human and animal subjects, which have demonstrated that the frontal cortex appears to be recruited during specific and targeted tasks in order to maintain motor performance with age (Goh 2011). Our study tested this same theory of ‘compensatory recruitment’ by assessing motor performance (across 2 age groups) after inducing ischemia in a region of the prefrontal cortex implicated in behavioural compensation, especially in an aged brain. By targeting the mPFC, in Experiments 1 and 2, we achieved our goal of inducing a non-motor stroke while also testing the theory of compensatory recruitment in the aged brain as compared to a younger cohort.

## 4.2 Summary of Results

In experiment 1, comparing young and middle-aged male Sprague Dawley, there were no cognitive or motor effects of mPFC ischemia (using an ET-1 model), as assessed using the RAM and the staircase test of fine motor performance. This finding supported the use of mPFC ischemia as a valid model of covert stroke, and laid the groundwork for Experiment 2: to further assess the role of the mPFC with respect to neuroplasticity and recovery of function in the aged brain. Importantly, although there was no difference in performance from baseline to post-mPFC ischemia, middle-aged rats showed decreased overall motor performance in both the staircase reaching test as well as the number of days to criteria in the RAM, as compared to the younger group.

In experiment 2, again comparing young and middle-aged male Sprague Dawley rats, there was no effect of previous mPFC ischemia in subjects who had a subsequent MCAo (again, using an ET-1 model) in either age group, as assessed using the staircase test of fine motor function. Unlike experiment 1, baseline performance in the middle-aged group was not significantly different from the younger group, yet post-stroke performance was significantly different between ages. The middle-aged group had significantly smaller post-stroke deficits following MCAo as compared to the younger group, and this was attributed to significantly smaller striatal infarct volumes.

## 4.3 Ischemic Model

The use of *bilateral* ischemic lesions was chosen, despite the fact that the overwhelming majority of strokes in the human population are unilateral, for a number of reasons. Rodents have less lateralized brain function than humans (Kolb, 1984), and in previous studies of prefrontal

damage in rat models, bilateral lesions have been used to induce detectable deficits (Fritts et al. 1998; Cordova et al. 2014, Livingston-Thomas et al 2015; Deziel et al., 2015). For this study, I sought to ensure that the target lesion would have a high probability of resulting in a detectable deficit, when followed by a motor stroke. Nonetheless, at the onset of Experiment 1, I planned to include a unilateral as well as a bilateral mPFC ischemia group as a comparison to accurately report the presence and potential gradient of behavioural motor or cognitive deficits in either group. Ideally there would have been a control group, a unilateral, and a bilateral mPFC ischemic group in both age groups (for a total of 6 groups), however this was not possible due to limitations in availability of appropriately aged animals. Instead, the pilot work included in Experiment 1 made use of the available subjects to appropriately power one experimental (bilateral mPFC ischemia) and one control group in the middle-aged group, with one control group and two experimental groups in the young group (unilateral and bilateral mPFC ischemia). By working within these constraints, I was able to demonstrate that there was no difference observed in the young animals in the staircase test or the RAM test, as compared to same-aged controls. There was also no difference within the middle-aged group following bilateral mPFC ischemia when compared to their own baseline performance or to their same age-matched controls. This finding was expected, as the function of the rodent mPFC is important for non-motor tasks and requires very specific cognitive assessments to reliably demonstrate cognitive deficits (Livingston-Thomas et al., 2015, Deziel et al., 2015). The data of Experiment 1 provided enough information to proceed with Experiment 2, by ruling out any motor deficits following mPFC ischemia. By demonstrating that there was also no significant difference in performance in the RAM, this also served to support the use of mPFC ischemia as a model of covert stroke where there is no "obvious " cognitive impairment.

#### 4.4 The Prefrontal Cortex

The functions of the prefrontal cortex in humans, as previously described, are complex and remain difficult to model in animals. It appears to play an important role in non-motor activities including attention set-shifting, working memory, decision making, and goal directed behaviour (Dalley et al., 2004; Livingston-Thomas et al., 2015; Vertes, 2006). Within our own lab we have examined the role of the mPFC and the effect of mPFC ischemia on attention set-shifting in an attempt to model executive dysfunction similar to the human deficits observed following small infarcts that interrupt connections to the prefrontal cortex. We have illustrated a significant difference in selective attention set-shifting (between stimulus dimensions, as opposed to within dimensions) following mPFC ischemia as compared to sham surgery (Cordova et al., 2014). This study took an in-depth look into cognitive function and did not demonstrate a difference in acquisition, selective attention shifting or perseveration. These results are in keeping with previous studies (Birrell & Brown, 2000; Livingston-Thomas et al., 2015; Deziel et al., 2015), and demonstrate the complexity of the prefrontal cortex while also suggesting that it has functional similarity to the primate dorsolateral prefrontal cortex, thus making it an important location for future studies seeking to model and assess the role of the lateral prefrontal cortex. Birrell and Brown showed that injury to the mPFC of Lister hooded rats yielded a very specific deficit in shifting of attention set while having no impact on acquisition of the attention set-shifting task nor on its reversal. This, again, is similar to the deficit observed in primates with lesions to the dorsolateral prefrontal cortex. More recent studies (Livingston-Thomas et al., 2015; Deziel et al., 2015) induced a focal ischemic infarct to the mPFC and, using a number of cognitive assessments, demonstrated that bilateral prefrontal ischemia resulted in significant changes in object recognition and behavioural flexibility. By using a number of

behavioural assessments, including an object recognition test, attention set-shifting, spontaneous alternation, Barnes maze and win-shift/win-stay tests, specific differences in performance were demonstrated following mPFC ischemia, allowing for a more sensitive detection of individual facets of cognitive impairment. These results support the choice of targeting the mPFC as an important area for executive cognitive function and future research into the role of the prefrontal cortex in more complex behaviours, including its role in neuroplasticity and compensation in an aging or an injured brain following an ischemic event. It also suggests why no cognitive deficits were observed in Experiment 1 of this study: in order to demonstrate a significant impairment in cognition due to mPFC ischemia, it is likely that more sensitive and demanding tests need to be employed.

As previously mentioned, no deficits in motor performance were observed following mPFC ischemia in young or middle-aged rats during Experiment 1, which was also replicated in the study by Livingston-Thomas and colleagues (2015). In order to reliably assess fine motor deficits, many previous studies have supported the use of the Montoya staircase skilled-reaching test as a sensitive assessment of motor impairment following cortical ischemia (Montoya et al., 1991; Biernaskie & Corbett, 2001; Windle et al., 2006; Clarke et al., 2009; Ploughman et al., 2009). Due to its sensitivity, ease of use, and well-established success as an indicator of motor impairment in our lab, it was chosen as the most appropriate assessment of motor performance.

#### **4.5 Middle Cerebral Artery Occlusion Model**

The decision to use the ET-1 MCA occlusion model to induce ischemia was in part due to its widespread use as a reliable model of ischemic motor stroke (Murphy & Corbett, 2009; Corbett et al., 2017). As reviewed by Windle and colleagues in 2006 and Durukan and

Tattisumak in 2007, and as was previously discussed in the introduction of this thesis, there are a number of experimental models by which to induce an ischemic stroke. International guidelines established by the Stroke Recovery and Rehabilitation Roundtable recommend that researchers employ models that are relevant to human stroke, minimally invasive, reproducible, and associated with an appropriate cost and effort (Corbett et al., 2017). Middle cerebral artery ischemic stroke induced by ET-1 significantly decreases cerebral blood flow in the territory supplied by the MCA, typically in a pattern that is similar to that achieved by direct surgical MCA occlusion, while being less invasive (Durukan & Tattisumak, 2007). By adjusting our usual coordinates for MCAo ischemia, which were used to produce motor stroke in young rats (Biernaskie et al., 2004; Ploughman et al., 2007; Windle et al., 2006), we had hoped to characterize an equally successful model of motor impairment in older Sprague-Dawley rats. Unfortunately, the pilot data derived from the very few older animals available prior to the start of my study provided false positive results (i.e. presence of motor deficits). In other words, the coordinates for the older rats were not optimal.

In retrospect, given the inherent variability in the MCAo model in young rats (Biernaskie et al., 2004) a better option would have been the injection of ET-1 into multiple cortical sites. A model using 2 forelimb cortical sites and one striatal site has been used on a number of occasions in our lab (Clarke et al., 2009; Windle et al., 2006). This may have allowed for more reliable and reproducible ischemic damage by directly targeting the motor cortex instead of the MCA which has a variable location deep within the perirhinal cortex. Previous studies using an aged model of ET-1 induced stroke in Sprague Dawley rats have had better success in reliable post-stroke motor deficits using cortical coordinates (Sun et al., 2016; Qu et al., 2015), suggesting that this model would be more reliable for future studies examining motor stroke in the aged brain.

In examining the infarct volumes for Experiment 1, there was no significant difference in infarct volume when comparing the bilateral mPFC injury between young and middle-aged rats. This was despite using the same depth of injection to target the mPFC, suggesting that there was no need to increase the depth of injection to account for increased skull thickness in the older group. We had carefully considered this when designing the initial experiment, as it was our experience that the more frontal aspect of the Sprague Dawley skull appeared to vary much less in thickness with age than the more lateral or temporal regions (ie. the lateral coordinate of the MCAo).

It would have also been helpful to have had more information about the location and extent of the injury for both the MCAo and mPFC ischemic models in this study. Statistical analysis of the infarct volumes revealed a significant difference in the striatal injury between the young and the middle-aged rats, but it is unclear as to where exactly this variability was most pronounced. For example, slight variations in antero-posterior or medio-lateral ischemic changes may have resulted in significant differences in behavioural outcomes, which were used to assess the primary outcome in this study. We chose not to exclude animals based on infarcts being too small, as there was no pre-determined cut-off for this value and it was difficult to arbitrarily assign an appropriate volume. With future studies, this additional information would be very helpful in documenting the success or assigning any need for improvement for similar ischemic models.

Importantly, there was very low mortality associated with the ET-1 surgical intervention. No animals were lost intra-operatively, and only one middle-aged animal that underwent ischemia died in the post-operative period (2.5% mortality). This is a low mortality rate, and is superior to that reported in other studies using an aged model (Soleman et al., 2010; Qu et al.,



2015). This may be due to the care and attention to detail during the intra-operative and post-operative period, limiting the number of poor surgical outcomes. Previous studies have suggested that deaths in the immediate post-operative period suggest more widespread diffusion of ET-1, expected to result in a larger infarct volume, or secondary to the accidental diffusion of ET-1 into the ventricles (Soleman et al., 2010).

#### **4.6 Limitations of Behavioural Tests of Motor Performance**

To date, the staircase apparatus has had limited use in *aged* rodent models, and this proved to be challenging to accommodate the larger, older animals. Despite the fact that we used larger Plexiglas® staircases there remained obvious difference in successful training rates between age groups. In our lab, the younger Sprague-Dawley rats learn to reach an average of ~17 pellets within a 10-14 day training period. In keeping with this, we have set a minimum training criteria average of 12 pellets with a standard deviation  $\leq 2$  pellets over a period of eight trials. Our successful training rate is more than 80% with our 3 month old Sprague-Dawley rats. In the first attempt at staircase in older rats, the successful training rate was markedly less in the middle-aged population (50% as compared to 83%). A study using an aged (20-23 months) rodent model of ET-1 induced stroke applied slightly less stringent training criteria, over a longer, 4 week period of training, and found an improved successful training rate of 79% (Soleman et al. 2010). However, their study also used Wistar rats as opposed to Sprague-Dawley, and previous studies have demonstrated a significant difference in staircase performance between various rat strains (Nikkah et al., 1998; Whishaw et al., 2003), making a direct comparison with my study difficult. Previous studies have also had more success in demonstrating motor deficits using other motor tasks, including the tapered beam walking test

and the cylinder test of forelimb asymmetry (Sun et al., 2016; Qu et al., 2015). It therefore remains a possibility that I may have found a more significant impairment in motor performance following MCAo if I had used other tests of motor performance.

#### **4.7 Challenges of an Aged Rodent Model**

Previous research has documented lower baseline performance of motor tasks as well as tasks of more complex cognitive function when using an aged rodent model as compared to younger cohorts (Soleman et al. 2010; Zhao et al. 2005; Qu et al. 2015). This is consistent with the results of this study with the middle-aged group performing more poorly than younger rats in the RAM *and* the staircase tests, even *prior* to ischemia. This may be due to age-related differences due to age in motor ability and dexterity, in overall health, and the size and structure of the behavioural testing apparatus, as was previously mentioned. Interestingly, this lower level of baseline performance in the staircase test was present in Experiment 1, but did not persist in Experiment 2, as is illustrated in Figure 2b as compared to Figure 5. This may have been secondary to an unconscious difference in training between both time points, as Experiment 1 was the first time our lab had used larger, middle-aged animals in staircase testing. We may therefore have unintentionally improved training efforts which led to improved success and an improvement in the average number of pellets from 15.3 to 17.3 in Experiment 1 and 2, respectively. There was no difference in the number of days used to train either group, and no conscious attempt made to improve training. Alternatively, the difference in baseline performance may reflect a difference in motivation from one group to the next, with middle-aged rats having more of a variation in motivation and therefore in performance. It is important to note that the poorer baseline performance was also demonstrated in the RAM in Experiment 1,

making decreased performance a consistent finding within this middle-aged group. It remains a possibility, however, that the middle-aged rats in Experiment 1 may have been less motivated for either behavioural test, as compared to the younger cohort, which was not the case in Experiment 2. This is an interesting possibility and warrants further research, because motivation has not been systematically studied in the context of stroke recovery, yet could have a major impact.

Researchers have preferentially used younger rodents in their models of stroke but as was outlined in the STAIR recommendations in 2009, researchers are being urged to maximize the applicability of their results to the human stroke experience by using older models of stroke in order to better reflect human stroke demographics. The problem remains, however, that this population of animals are more challenging to incorporate into experimental models due to a number of rodent age-related health issues (e.g. respiratory illness, tumour formation) not to mention increased costs. In this study we had a number of animals who died prior to the study start-date, as they arrived at our centre at 5-6 months of age and had to be “aged” another 6 months before beginning the study. We did not assess the cause of death in these animals, but previous publications have described rats with acute deterioration in health, ultimately attributable to pituitary tumours or abdominal masses felt to be secondary to advanced age (Soleman et al., 2010; Corbett lab, unpublished data). This loss of subjects unfortunately limited the final number of subjects in my middle-aged group.

#### **4.8 Conclusion**

In conclusion, this study did not support the hypothesis that the mPFC plays a crucial supportive role in post-stroke motor recovery in either young or aged Sprague Dawley rats, as assessed using the staircase test. As has been previously discussed, this may be secondary to a

number of factors which have been described in the preceding sections 4.3-4.7, including limitations of the behavioural test employed, the challenges of working with an aged rodent model, and the imperfections of the chosen ischemic model. After careful analysis of the results, the difference in infarct size between age groups (as a reflection of ischemic model), may explain the negative results. The smaller striatal infarct volume in the middle-aged subjects precludes meaningful comparisons to the successful strokes achieved in younger rats. Future work needs to focus on methods to attain similar amounts of ischemic damage across different age groups.

Importantly, however, there were several important findings and implications raised by this study. To our knowledge, this is one of the first studies to describe the effect of prefrontal stroke in a rodent model of recurrent stroke, and is among only a small number of studies to specifically examine its effect on motor performance. This model could be used in a similar fashion to explore the contribution of additional brain structures in both the contralesional and ipsilesional cortex to post-stroke recovery. An interesting finding in the present study was that the pre-stroke staircase and RAM performance of the middle-aged rats used in Experiment 1 was significantly lower than that of their younger counterparts. It is presently unclear whether this is due to differences in motivation or some altered motor capability that might be revealed by using pre-and post-stroke reaching kinematics in young and older animals (Corbett et al., 2017). Such an analysis could reveal potential age-related differences in compensation versus restitution between young and older animals, with older animals adopting more compensatory movement patterns. While speculative, such questions have not previously been addressed and would help to interpret post-stroke performance changes across the aging continuum. Other avenues of research need to focus on further developing rodent models of stroke in older animals. For

example, given the difficulties using the staircase test in old rats that reach 700-800 g in weight it may be preferable to use the single pellet reaching task instead (Whishaw & Pellis, 1990).

The growing concerns over translational failure in stroke and other fields of medicine require that animal models capture more aspects of the clinical population including disease comorbidity, aging and sex differences (Fisher et al., 2009; Corbett et al., 2017, Bosetti et al., 2017). The present thesis represents a step in this direction.

## **5. Figures**

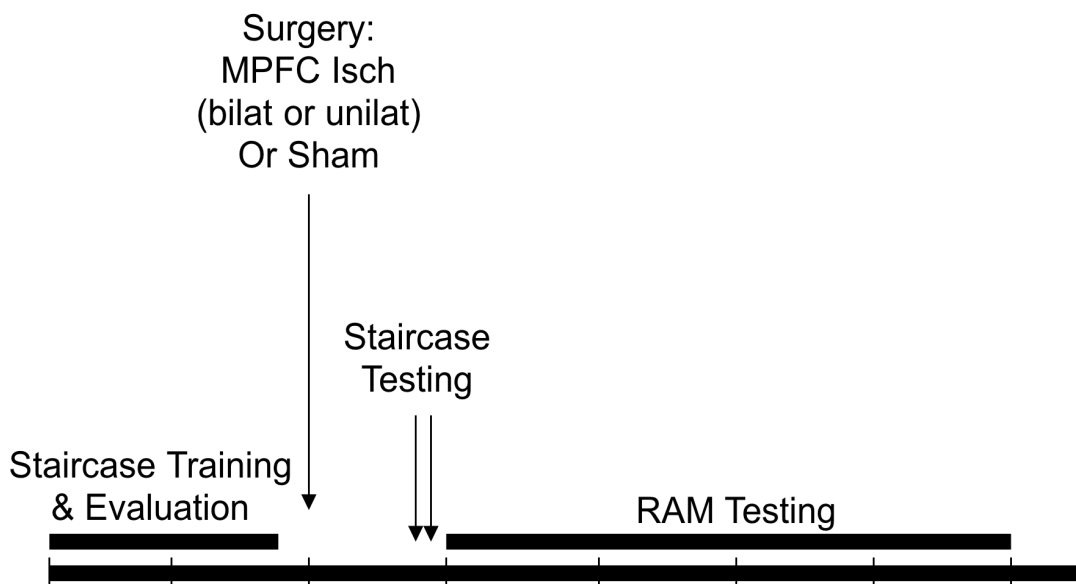
Figure 1a: Timeline for Experiment 1. Each vertical dash represents one week in the study.

Staircase training, followed by baseline staircase testing, was completed two days prior to mPFC ischemia or sham surgery. Subjects included young animals that underwent bilateral mPFC ischemia ( $N = 8$ ), unilateral (left sided) mPFC ischemia ( $N = 7$ ), and sham surgery ( $N = 7$ ); plus middle-aged animals that underwent bilateral mPFC ischemia ( $N = 8$ ), and sham surgery ( $N = 8$ ). Animals were then tested in the staircase apparatus on post stroke day 5 and 6, followed on day 7 by radial arm maze testing for a maximum of 4 weeks.

Figure 1b: Timeline for Experiment 2. Each vertical dash represents one week in the study.

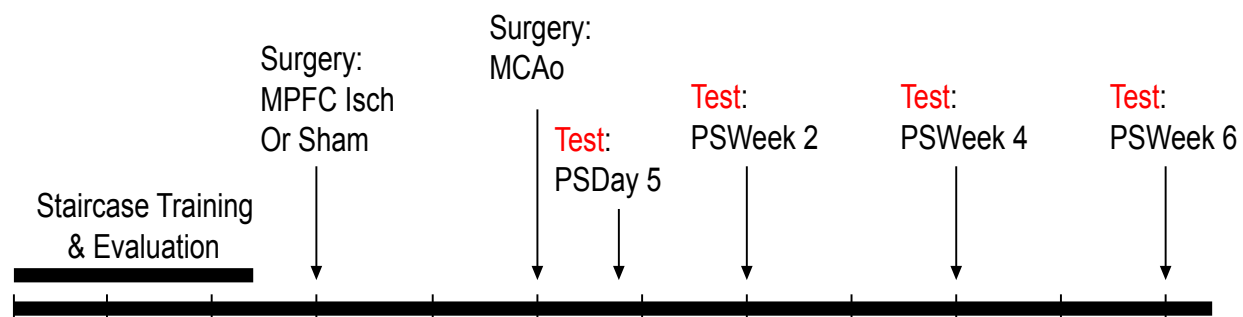
Staircase training, followed by baseline staircase testing, was completed two days prior to mPFC ischemia or sham surgery. Two weeks following the initial surgery, all animals then underwent MCAo, followed by post-stroke staircase testing on day 5 and 6, week 2, week 4, and week 6.

Figure 1a:



Bilat: bilateral; Unilat: unilateral; RAM: radial arm maze

Figure 1b:



Isch: ischemia; MCAo: middle cerebral artery occlusion; MPFC: medial prefrontal cortex; PS: post-stroke

Figure 2a: Experiment 1 - Staircase Performance: Young Group. Each point represents the average number of pellets retrieved from the staircase below the contralateral limb to the ischemic hemisphere (maximum of 21 pellets). There was no significant difference in the number of pellets retrieved at pre- or post-surgery testing, therefore confirming the absence of motor deficits in this rodent model. Error bars represent the SEM.

Figure 2b: Experiment 1 – Staircase Performance: Young and Middle-Aged Group. Each point represents the average number of pellets retrieved from the staircase below the contralateral limb to the ischemic hemisphere (maximum of 21 pellets). Similar to Figure 2a, there was no significant difference between pre and post-stroke performance in the middle-aged group. There was, however, a significant difference in performance due to age at both time points, where middle-aged animals were worse at both time points ( $*p < .001$ ).



Figure 2a:

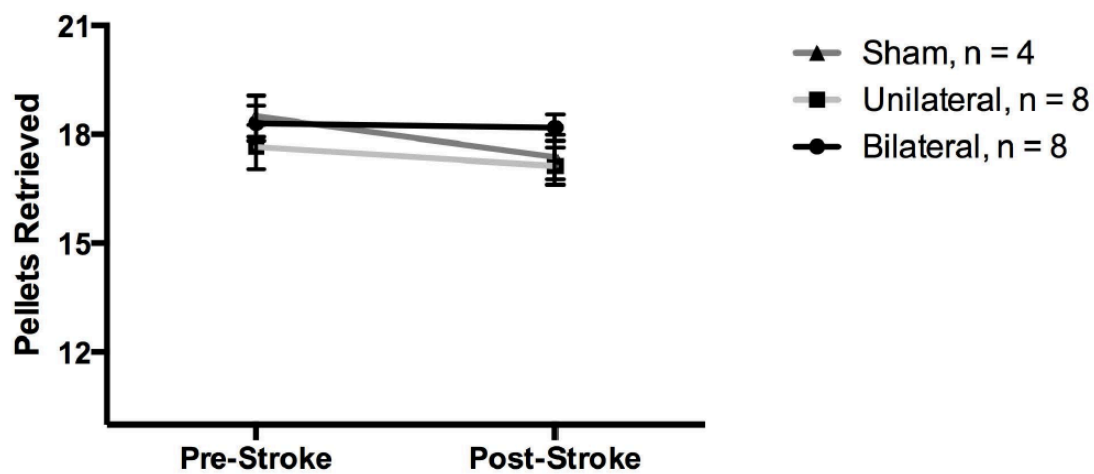
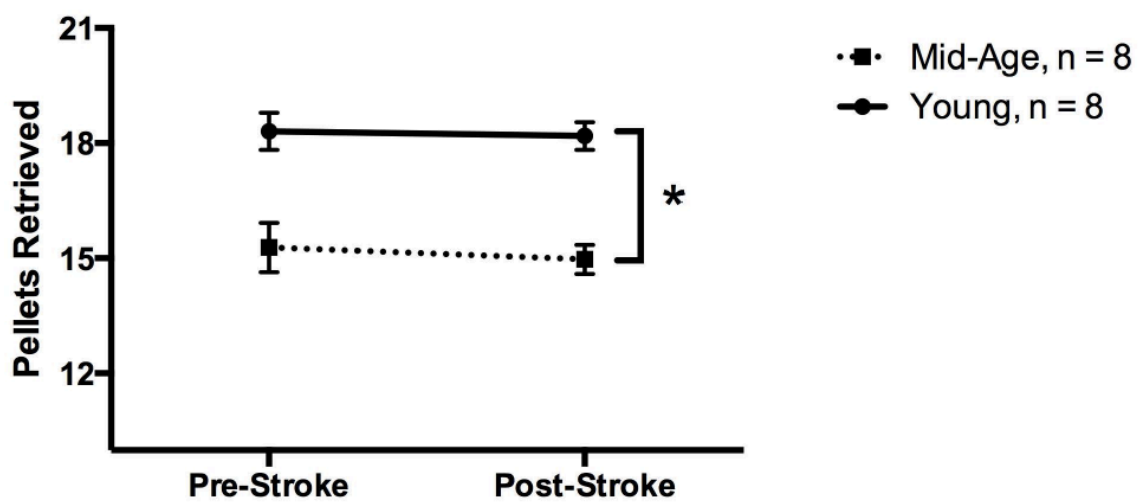


Figure 2b:



Mid-age: middle-aged

Figure 3a: Experiment 1 – Radial Arm Maze Performance: Young Group. Each bar represents the average number of days to criteria. There were no significant impairments in radial arm maze performance, for either unilateral or bilaterally injured young rats as compared to the sham group. Error bars represent the SEM.

Figure 3b: Experiment 1 – Radial Arm Maze Performance: Young and Middle-Aged Group. Each bar represents the average number of days to criteria. There were no significant cognitive impairments as a result of mPFC ischemia relative to sham (stroke x age,  $p = .85$ ; stroke,  $p = .97$ ). There was, however, a significant effect of age similar to that seen in the staircase task, whereby middle-aged rats were significantly impaired in their ability to learn the task as compared to younger rats ( $*p < .001$ ). Error bars represent the SEM.

Figure 3a:

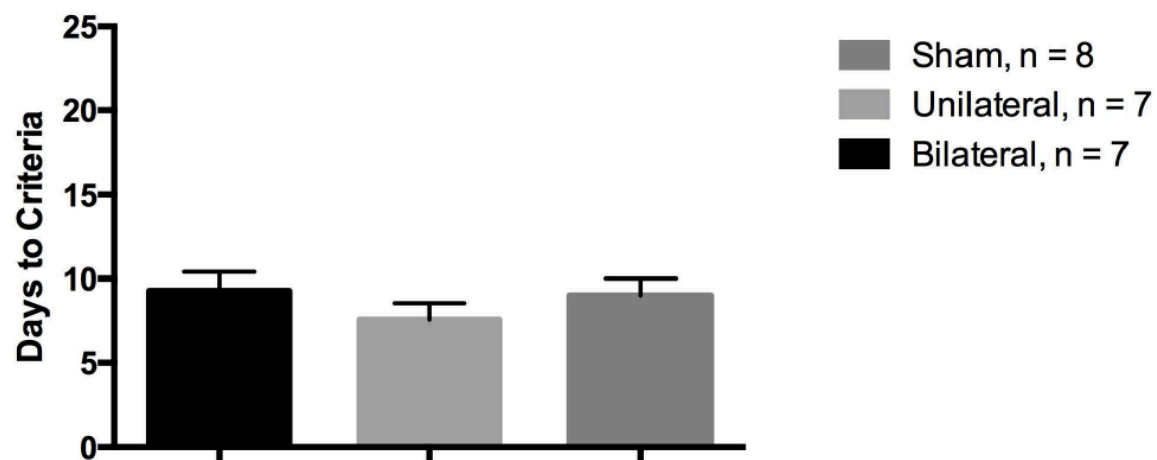
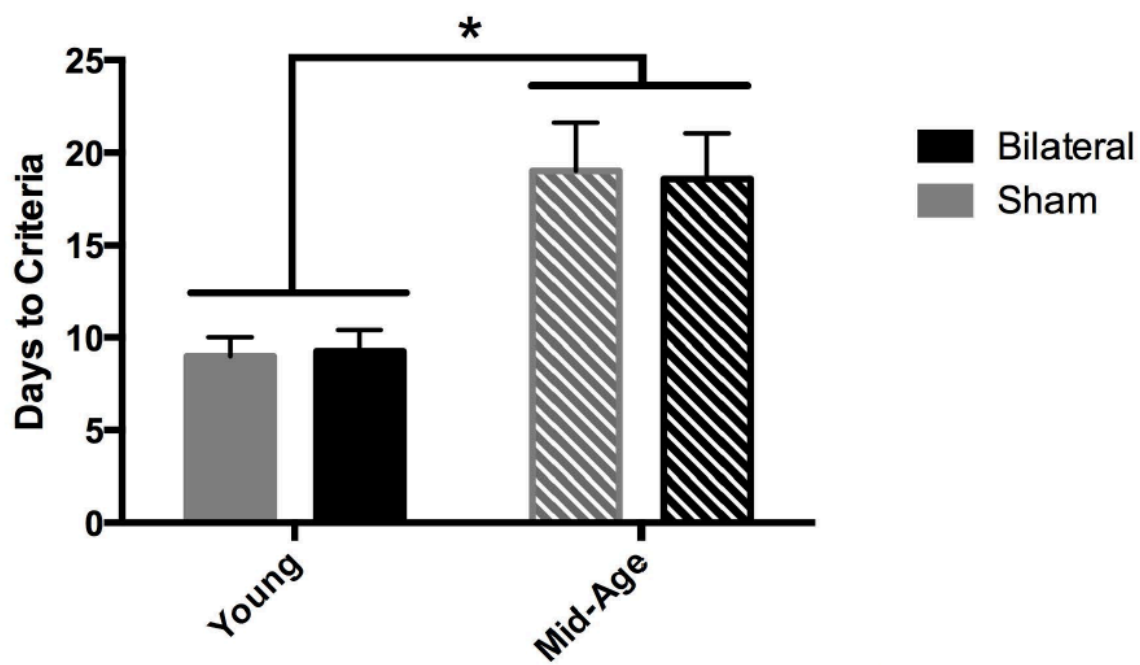


Figure 3b:



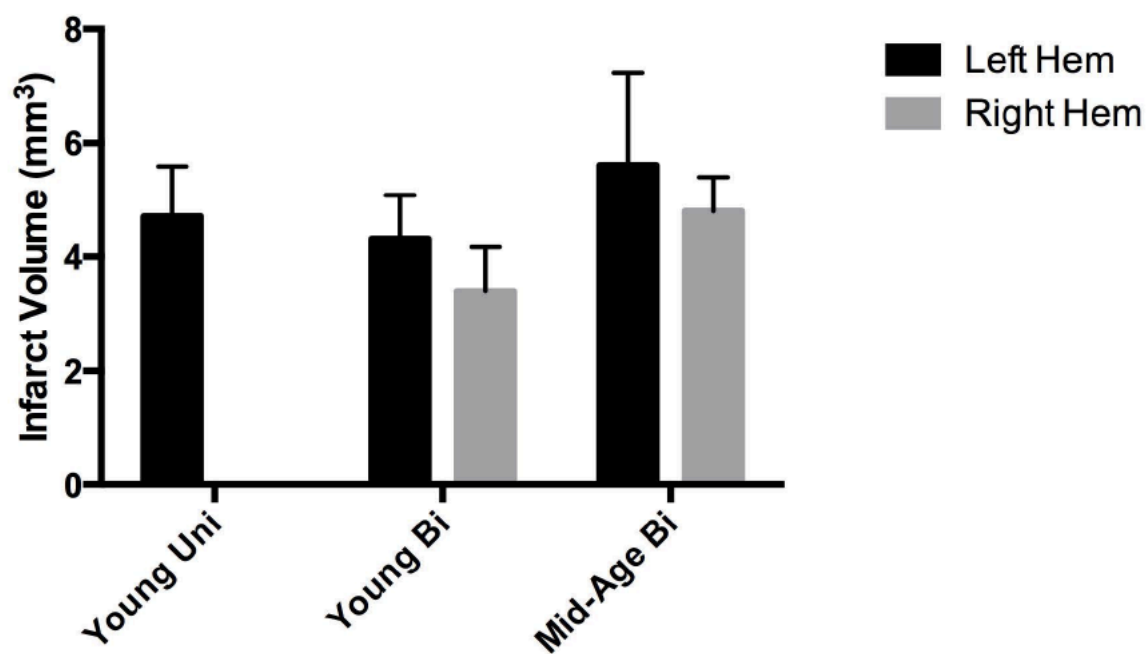
Mid-age: middle-aged

Figure 4a: Experiment 1 – Infarct Volumes: Young and Middle-Aged Hemisphere Comparisons.

Each bar represents the average infarct volume in those who underwent either unilateral or bilateral mPFC ischemia. There was no significant difference between groups. No significant differences were observed between damage to the left or right hemisphere for animals that underwent bilateral PFC ischemia in either age group, and no difference in infarct volume of either hemisphere when compared to the young group following unilateral ischemia. The young animals that underwent unilateral mPFC ischemia had no injury to the contralateral hemisphere. Error bars represent the SEM.

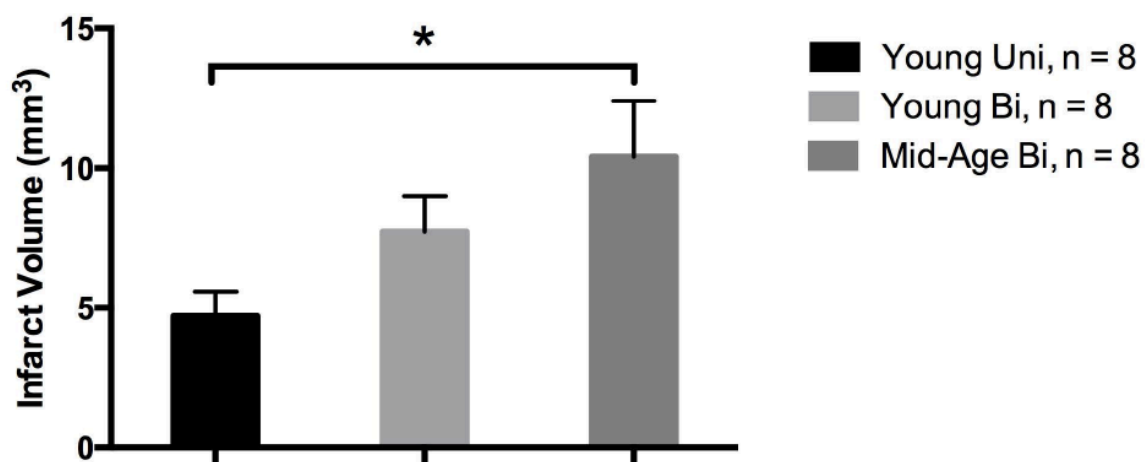
Figure 4b: Experiment 1 – Infarct Volumes: Young and Middle-Aged Total Volume. The young, unilateral mPFC group had significantly less total injury than the middle-aged bilateral mPFC ischemic group ( $*p = .03$ ). There was no significant difference in infarct volume between the young bilateral mPFC ischemic group and the middle-aged bilateral mPFC ischemic group. There was also no significant difference between the young and middle-aged bilaterally injured groups. Error bars represent the SEM.

Figure 4a:



Bi: bilateral; Hem: hemisphere; Mid-age: middle-aged; Uni: unilateral

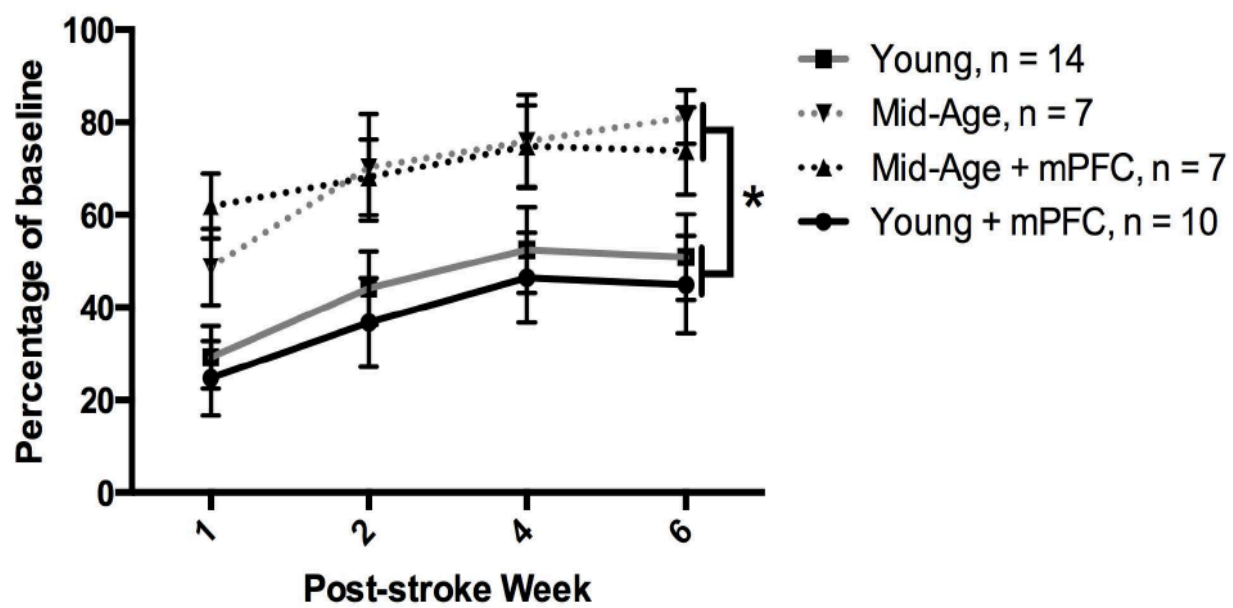
Figure 4b:



Bi: bilateral; Mid-age: middle-aged; Uni: unilateral

Figure 5: Experiment 2 – Staircase Performance: Young and Middle-Aged Group. As described, all groups underwent MCAo injury, and significant differences between ages were observed ( $*p = .014$ ). Interestingly, middle-aged rats were significantly less impaired than young rats after MCAo, regardless of mPFC injury. Additionally, a significant effect of time was observed ( $p < .001$ ), demonstrating that all groups increased their performance across weeks by the same degree. Error bars represent the SEM.

Figure 5:

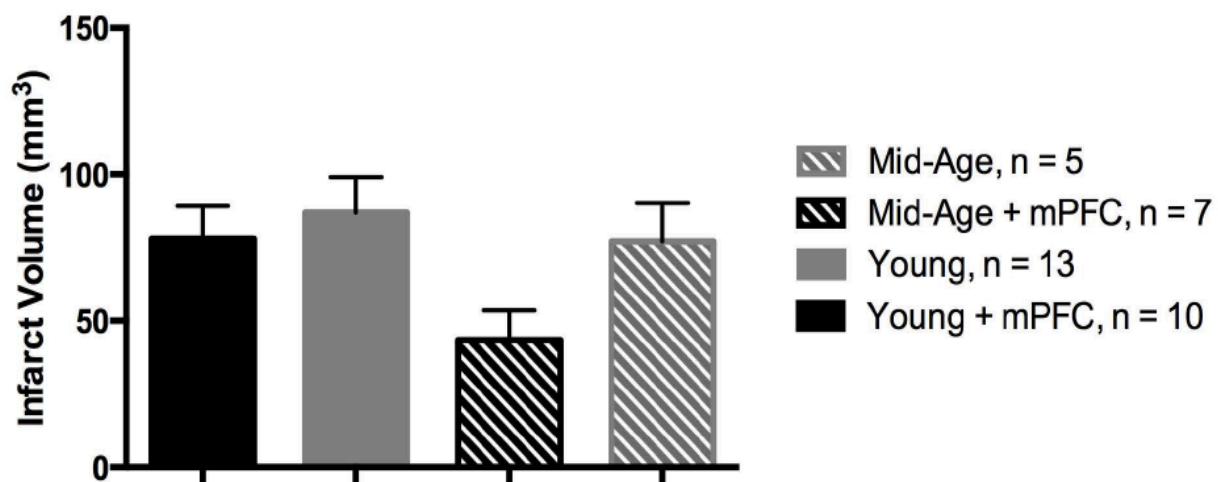


Mid-age: middle-aged; mPFC: medial prefrontal cortex

Figure 6: Experiment 2 – Total Infarct Volume: Young and Middle-Aged Group. Each bar represents the average total infarct volume in those who underwent MCAo with or without mPFC. There were no significant differences between any group with respect to total infarct volume. Error bars represent the SEM.



Figure 6:

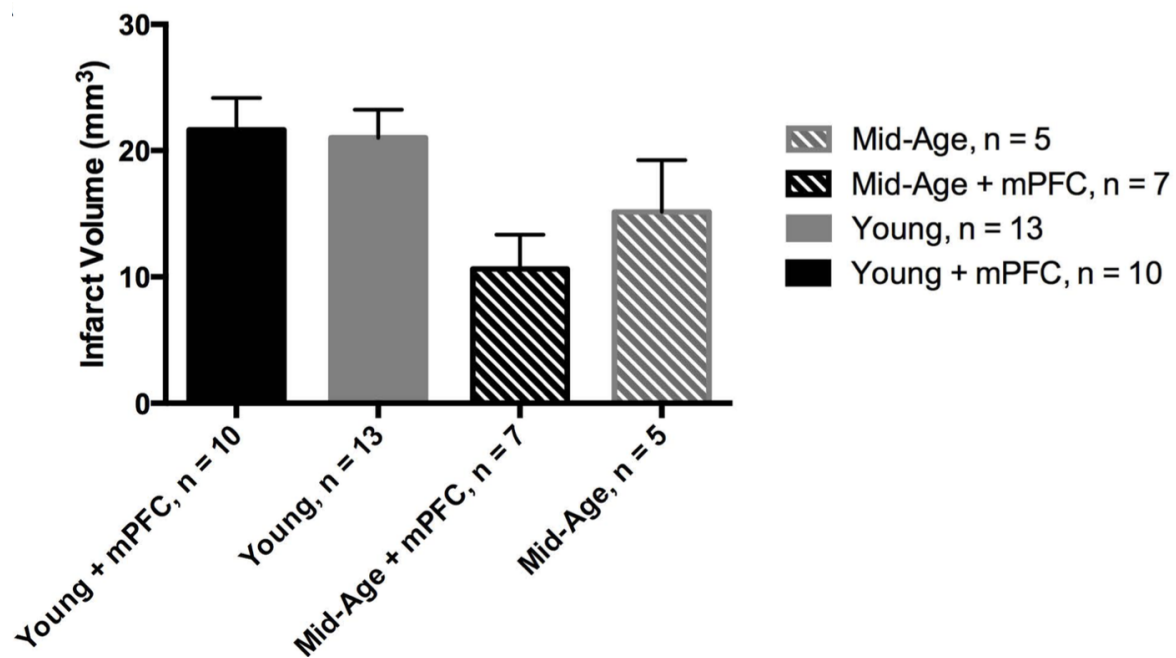


Mid-age: middle-aged; mPFC: medial prefrontal cortex

Figure 7a: Experiment 2 – Striatal Infarct Volume. Each bar represents the average volume of striatal infarct in those who underwent MCAo with or without mPFC. There was no significant stroke by age interaction, and no difference between groups. Error bars represent the SEM.

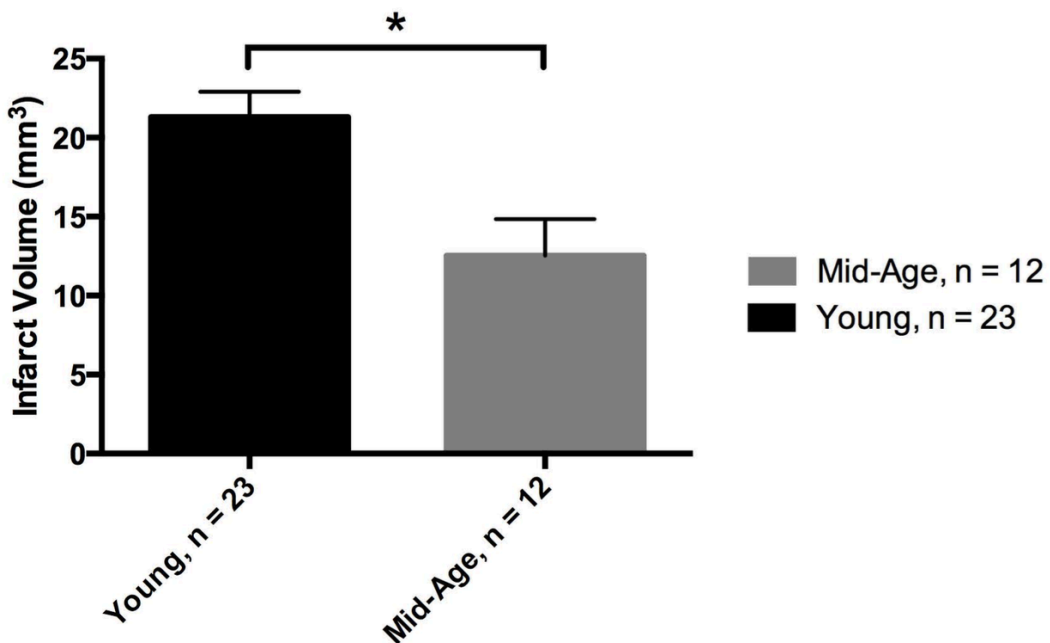
Figure 7b: Experiment 2 – Striatal Infarct Volume, Represented By Age. Each bar represents the average volume of striatal infarct in those who underwent MCAo with or without mPFC. There was a significant effect of age, as middle-aged rats had significantly smaller striatal infarct volumes than younger rats, regardless of whether a prior mPFC injury had occurred or not. Error bars represent the SEM.

Figure 7a:



Mid-age: middle-aged; mPFC: medial prefrontal cortex

Figure 7b:



Mid-age: middle-aged

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